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StPUB17, a novel potato UND/PUB/ARM repeat type gene, is associated with late blight resistance and NaCl stress

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ABSTRACT

StPUB17, a novel UND/PUB/ARM repeat type gene, was isolated from leaves of potato (*Solanum tuberosum* L.) clone 386209.10 using the rapid amplification of cDNA ends strategy with the primers designed according to a potato EST fragment up-regulated by *Phytophthora infestans*. *StPUB17* was confirmed intron-free by comparison of the cDNA and genomic DNAs. The RT-PCR analysis showed that *StPUB17* was constitutively and differentially expressed in all tissues, and was significantly induced in detached leaves subjected to *P. infestans*, signal molecules such as salicylic acid, methyl jasmonate, ethylene, abscisic acid and wounding. The gene expression was also strongly up-regulated when *in vitro* plantlets were exposed to high (40 °C) and low (4 °C) temperatures and dehydration induced by polyethylene glycol and NaCl. The function of *StPUB17* was further clarified by silencing it in potato using RNAi-based post-transcriptional gene silencing (PTGS). The results demonstrated that *StPUB17*-silenced plants exhibited more susceptible to the infection of *P. infestans* and more sensitive to the stress of NaCl. Present data indicated that *StPUB17* is a gene harboring broad-spectrum responses to both biotic and abiotic stresses in the potato and may play crucial roles in late blight resistance and salt tolerance of the crop.

1. Introduction

Phytophthora infestans is an oomycete (Phylum *Oomycota*, Order *Peronosporales*, Family *Pythiaceae*), the causal agent of late blight disease of potato which is distributed worldwide. With immigration of A2 mating type late blight has produced significant impacts on potato production [1,2]. Two major types of late blight resistance are recognized in potato: vertical resistance (also called race specific or monogenic resistance) and horizontal resistance (also called race nonspecific, filed or polygenic resistance). The vertical resistance is a form of localized programmed cell death known as hypersensitive response (HR) that leads to total cessation of the infection process or development of small lesions with limited sporulation [3,4]. *P. infestans* is highly variable and all new potato cultivars with vertical resistance eventually succumb to a rapid evolution of the pathogen races. However, the plant horizontal resistance has polygenetic inheritance and quantitative phenotype and is thus considered to be more durable and reliable compared to the vertical resistance [5,6]. In order to reveal molecular events associated with horizontal resistance to late blight, a cDNA library highly enriched for horizontal-resistance-related genes was constructed by the suppression subtractive hybridization (SSH) technique [7]. Among the first 150 clones identified as positive, one fragment reveals significant sequence similarity to a tobacco (*Nicotiana tabacum*) Avr9/Cf-9 rapidly elicited protein 276 (*ACRE276*) gene that encodes an U-box protein with ARM repeat-domains and is a bona fide E3 ubiquitin ligase [8].

Plants defend themselves against potential invading pathogens by activating a battery of defense mechanisms, in which numerous defense-related genes are coordinately expressed. In fact, most of the studies that have been made so far take advantage of an approach based on identification of novel proteins resulted from induction of gene expression during defense responses [9]. On the other hand, recent studies have also shown that degradation of proteins is one of most important biochemical and physiological

Abbreviations: ABA, abscisic acid; ACRE, Avr9/Cf-9 rapidly elicited; ARM, armadillo; ETH, ethylene; HR, hypersensitive response; IE, infection efficiency; LGR, lesion growth rate; MeJA, methyl jasmonate; ORF, open reading frame; PEG, polyethylene glycol; PTGS, post-transcriptional gene silencing; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; RT-PCR, reverse transcription polymerase chain reaction; PUB, plant U-box protein; SA, salicylic acid; UND, U-box N-terminal domain.

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events that play critical roles in regulating almost all aspects of cellular processes [10]. Ubiquitin-mediated protein degradation has gained even more attention because of its specificity of selectively recognizing and degrading the target protein [11,12].

Ubiquitination is an important mechanism for post-translational modification of cellular proteins [13,14], in which ubiquitin is initially activated and transferred to an ubiquitin-conjugating enzyme (E2) by an ubiquitin-activating enzyme (E1) to form E2-ubiquitin complex. The specificity of ubiquitination is controlled by E3 ubiquitin ligases which selectively recognize the protein substrate and catalyze ubiquitin transfer from the E2-ubiquitin complex to the target protein for breakdown [15].

The E3 ubiquitin ligases can be categorized based on the mechanism of action and presence of specific domains [16,17]. The U-box domain, a peptide chain that contains approximately 70 amino acid residues, is suggested to be a conserved domain found in diverse isoforms of E3 ubiquitin ligase in eukaryotes [18]. The armadillo (ARM) repeats are 42-amino acid tandem repeated sequence domains and mediate protein-protein interactions [17]. The Plant U-box/ARM repeat proteins (PUB/ARM proteins), suggesting a plant-specific group of E3 ubiquitin ligases defined by the presence of the U-box followed by an ARM repeat-domain, comprise the largest group with 41 and 43 predicted members in the *Arabidopsis* and rice (*Oryza sativa*) genomes, respectively [19]. Within them, there are 16 members in each species that contain a previously identified domain termed UND (U-box N-terminal domain) which is conserved in plants [17,19]. However, the functions of many UND/PUB/ARM repeat proteins remain unknown.

A rice UND/PUB/ARM repeat protein, OsSPL11, was thought to be a negative regulator of cell death and defense [20]. In contrast, a similar protein in the *Arabidopsis*, AtPUB17, was considered to be positive regulator of cell death and defense responses [8,21]. In addition, several expressed UND/PUB/ARM repeat proteins were identified from other plant species. Yang et al. (2006) speculated that ACRE276 and NtPUB4 of tobacco (*N. tabacum*) were involved in Avr9/Cf-9 rapidly elicited defense and tobacco development signaling, respectively. BnARC1 from *Brassica* was reported to be required for the self-incompatibility (SI) response [22] and BgBG55 from mangrove (*Bruguiera gymnorrhiza*) was found to have a transient increase in expression in response to salt treatment [23]. The information available suggests that UND/PUB/ARM repeat proteins may have different functions in diverse biotic and/or abiotic responses and physiological processes in specific plant species. Here we report a novel UND/PUB/ARM repeat gene from potato, *StPUB17*, which was shown to be inducible by *P. infestans* and various abiotic elicitors through RT-PCR analysis, and its function suggests that it plays crucial roles in late blight resistance and salt tolerance by RNAi-based post-transcriptional gene silence.

2. Materials and methods

2.1. Plant materials and bacterial strains

Potato (*Solanum tuberosum* L.) cultivars 386209.10 (R-gene-free, horizontal resistance to late blight, kindly provided by the International Potato Center (CIP), Lima, Peru), E-potato 3 (vertical resistance to late blight) and Zhuanxinwu (susceptible to late blight) were used for the experiments.

In vitro plantlets of the three potato cultivars were propagated in sterile culture boxes containing MS medium [24] supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar and raised in growth chamber under a 16 h light/8 h dark regime at 20 °C. Four- to 5-week-old plantlets were transplanted into pots in a greenhouse under the normal condition, the third to fifth fully expand leaves (counted from the top) were used for the inoculation of *P. infestans*. Microtubers for potato transformation were induced from the nodal segments of *in vitro* plantlets according to the procedure of Liu et al. [25].

Escherichia coli DH5α was cultured by standard techniques [26] and used for cloning and propagation of recombinant plasmids. *Agrobacterium tumefaciens* strain LBA4404 was cultivated in YEB medium [26].

2.2. Phytophthora infestans inoculation

A mixture of isolates (H1, W8, Ljx18 and ZY15) of *P. infestans* was cultured on potato tuber slices at 18 °C according to Fang [27]. For each experiment, freshly produced sporangia were collected in sterile water from the tuber slices 6–7 days after the culture, and incubated at 4 °C for 1–2 h to release zoospores. The concentration was adjusted to 5×10^4 ml^{-1} zoospores for the potato detached leaves inoculation [28,29]. Sterile water was applied to control leaves instead of zoospores. Three replicates, eight leaflets in each, were employed.

2.3. Treatment with abiotic elicitors and environmental stresses

The detached leaves of potato 386209.10 were respectively sprayed with abiotic elicitors, 2 mM salicylic acid (SA, pH 6.5), 50 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA) or 200 μl l^{-1} ethylene (ETH) (Sigma–Aldrich, St. Louis, USA). SA, MeJA and ABA were dissolved in 0.1% ethanol. The control leaflets were treated in the same way by spraying with 0.1% ethanol or distilled water based on the solvent used for each treatment. For mechanical wounding, the leaves were injured by punching four cuts on each side of the middle vein with a 1 ml syringe without a needle according to Tian et al. [30]. All of the treated samples were covered with a transparent polyethylene plastic bag to maintain humidity.

Four-week-old *in vitro* plantlets of potato 386209.10 were treated with various environmental stresses. For cold and heat stress treatments, plantlets were placed at 4 or 40 °C on a thermostatically controlled heating block, respectively. To impose dehydration stress, the plantlets were removed from the MS medium and transferred to Petri dishes with lids covered after removing excess water with facial tissues, a piece of filter paper was placed underneath the plantlets to facilitate water evaporation. The osmotic and salt treatments were performed by adding 20 ml 20% PEG (polyethylene glycol) and 200 mM NaCl solution into the culture boxes, respectively. The controls were treated in the same way but with distilled sterilized water.

The treated samples were collected after different times as indicated and frozen in liquid nitrogen and maintained at −70 °C until use.

2.4. RNA isolation and first strand cDNA synthesis

Total RNAs were extracted from various tissues and stress-treated leaves using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The extracted RNA was treated with DNase I (Promega, WI, USA) at 37 °C for 15 min to remove the remaining genomic DNA. The first strand cDNA was synthesized by oligo-d(T)$_{16}$ primer and reverse transcriptase Superscript-II RT (GIBCO-BRL) following the manufacturer's protocol.

2.5. Cloning and sequence analysis of StPUB17 gene

To obtain full-length cDNA of potato *StPUB17* gene, a pair of gene specific primers (GSP1: 5′-CAGACATTGCTCTTTACAGGAACA-3′ and GSP2: 5′-ATAATACTGGAACTGAAATGG ACC-3′) were designed according to the EST sequence [7] and synthesized by Biocolor

(Shanghai, China). The total RNA purified from potato 386209.10 leaves inoculated with *P. infestans* was used to synthesize single-stranded cDNA, and the full-length cDNA was obtained according to the strategies of RACE combined with overlap extension PCR methods [30]. The single PCR products were separated on 1% agarose gel and purified with Gel Extraction Kit (HuaShun, Shanghai, China) according to the manufacturer's protocol. The purified products were then cloned into the pMD18-T vector (TaKaRa) according to the manual and transformed into *Escherichia coli* strain DH5α using the freeze-thaw method [26]. Positive clones characterized by blue/white screening were confirmed by sequencing (Biocolor, Shanghai, China).

Primers (5′-ACAAGAAAAGGGTTGGAGTTGATTA-3′ and 5′-TACAGGCAAAACTAAGA ACAAACGA-3′) were designed based on the 5′ and 3′ cDNA sequences to amplify the full-length genomic fragments of potato *StPUB17* gene. Genomic DNAs were extracted from young leaves of 5-week-old greenhouse-grown plants of potato cvs. 386209.10, E-potato3 and Zhuanxinwu. Similarly, PCR products were cloned into the pMD18-T vector and sequenced on both strands as described above. Three positive clones of each template were sequenced to confirm product specificity and obtain a consensus sequence.

Similarity searches were carried out using BLAST at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments and phylogenetic tree construction were performed by the ClustalW program of DNAstar software (LaserGene, Madison, WI, USA).

2.6. RT-PCR analysis

The concentration and quantification of the total RNA of each sample was measured by spectrophotometry and the PCR cycle numbers were optimized to determine the amplification (26–30 cycles in a 25 μl system). The PCR reactions (corresponding to each treatment) were assayed simultaneously in separate tubes using the identical cDNA aliquots (1 μl) of the RT reaction products with the gene specific primers: 5′-TTCTTGGCGCCGGTGGATCTGACA-3′ and 5′-ACGGCAAATCTGAAGAACCCCTAC-3′. To avoid amplification

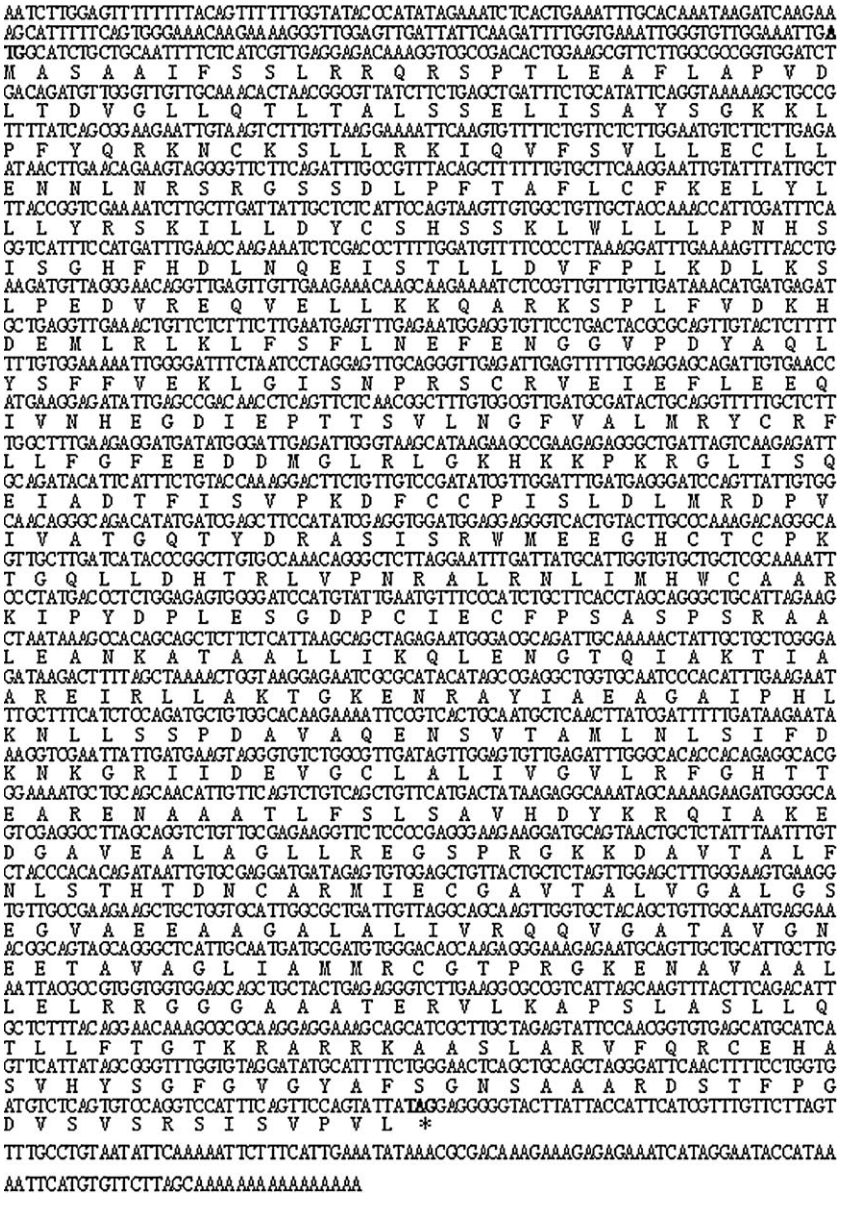

Fig. 1. Nucleotide and deduced amino acid sequences of *StPUB17*. The deduced amino acid sequence is shown underneath the corresponding nucleotide sequence (GenBank accession no. EF091878). The start codon ATG and the stop codon TAG are boldface, the stop codon is indicated with "*". The gene specific primers are underlined.

of genomic contaminations and pseudo positive product, total RNA was used as a template during all RT-PCR analysis. Moreover, the constitutively expressed potato *Actin* gene was used as an internal control with the primer pairs designed based on *S. tuberosum PoAc58* gene (GenBank accession no. X55749.1).

RT-PCR was performed using the following program: denaturation at 94 °C for 5 min; 26–30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s; a final extension step at 72°C for 5 min and ending at 4°C. Single bands of PCR products were confirmed on agarose gel. Relative expression of *StPUB17* mRNA was *StPUB17* to *Actin* ratio and analyzed by densitometric measurement using Quantity One 1-D Analysis Software (Bio-Rad, USA). The mean density for each band was calculated and expressed as the ratio of *StPUB17* to *Actin*. Error bars indicate standard deviation values of RT-PCR analyses. Each experiment was repeated three times independently on the same RNA sample.

2.7. Construction of plant expression vector and transformation of potato

A 370 bp fragment of *StPUB17* gene was used for constructing the RNA interference (RNAi) vector for the gene silencing in potato. This fragment was amplified with the cDNA template using Gateway attB1- and attB2-extended primers: attB1 + *StPUB17*: 5′-AAAAAG-CAGGCT(CAGATACATTCATTTCTGTACCAAA)-3′ and attB2 + *StPUB17*: 5′-AGAAAGCTGGGT(ATCTGCGTCCCATTCTCTAGCTGCT)-3′. The 12 bp upper-case characters correspond to the attB1 and attB2 primer sequences. Subsequently, the fragment was recombined into the gene silencing vector pHGRV by BP reaction according to the manufacture's instruction (Invitrogen, Carlsbad, CA, USA). The recombinant plasmid was verified by sequencing and then introduced into the *A. tumefaciens* strain LBA4404 *via* the freeze-thaw procedure [31].

Arobacterium-mediated transformation of potato microtubers was carried out as described previously [32]. Re-generated shoots were rooted on MS medium containing 200 μg ml^{-1} kanamycin and 250 μg ml^{-1} carbenicillin and the transgenic plants were confirmed by the PCR with specific primers of *NPT* II gene.

2.8. Analysis of StPUB17-RNAi transgenic plants for P. infestans resistance and NaCl tolerance

The assay for disease resistance against *P. infestans* was performed using the method of Vleeshouwers et al. [28]. Leaves from 6-week-old plants of transgenic lines and non-transgenic controls were inoculated with 10 μl of 5×10^4 ml^{-1} freshly produced zoospores, and maintained at 18 °C, 95–98% relative humidity and 16 h light/8 h dark photoperiod provided by fluorescent lamps. When late blight symptoms appeared, the largest length and width (perpendicular to the length) of each lesion were measured on days 3, 4, and 5 days after inoculation, and the lesion area was calculated using the formula for ellipse ($A = 1/4 \times \pi \times$ length $\times$ width). The radius of the lesion was then obtained by square-root transformation of the area and it was used in a linear function against time to estimate the lesion growth rate (LGRs, mm d^{-1}), i.e., the regression coefficient b of the formula, $r = a + bt$, where r is the radius of the lesion, a is a constant, and t is duration of the inoculation in days. The infection efficiency (IE) was calculated as the percentage of successful inoculations (i.e., percentage of growing lesions relative to the total number of inoculations) for each sample. Three replicates were setup with eight leaflets in each.

To determine the level of salt tolerance the shoot tips of transgenic plantlets and control (non-transformed E-potato 3) were respectively cultured on MS medium supplemented with 0, 100 or 200 mM NaCl. The cultures were maintained at a temperature of 20 °C and a 16 h light/8 h dark photoperiod. After 15 days of the salt treatment, the plantlets were harvested, and shoot length, fresh weight, rooted and decayed (the stem-end of the plantlet is decayed and loses capacity to form roots) numbers of plantlets were determined. Each treatment contained sixteen plantlets with six replicates.

3. Results

3.1. The StPUB17 gene cloning and sequence analysis

Combining reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA end (RACE) techniques using degenerate primers and gene specific primers, the full-length cDNA of *StPUB17* gene was obtained from the mRNA isolated from potato 386209.10 leaves inoculated with *P. infestans*. This sequence is of 2483 bp (GenBank accession no. EF091878), containing a 157 bp 5′ untranslated region (UTR), a 2175 bp open reading frame (ORF) encoding a protein of 724 amino acids, and a 151 bp 3′ non-coding region (NCR) (Fig. 1).

In order to find out whether the *StPUB17* gene has intron(s), the 5′ and 3′ coding region primers were designed to amplify the genomic sequence from potato cvs. 386209.10, E-potato 3 and Zhuanxinwu. Comparison between the cDNA and genomic DNAs revealed that the sequences maintained high levels of conservation in terms of sequence length and nucleotide composition, and all the gDNAs from the three cultivars seemed to be composed of a single exon without intron (see supplementary Fig. S1).

Searching BLAST indicated that the cDNA sequence showed the greatest match (96% identities) to *Solanum lycopersicum* chromosome 2 clone C02SLm0096H01 gene (GenBank accession no. AC226521) and C02SLe0011K05 gene (GenBank accession no. AC215445), and has 91% identities in the coding region to *N. tabacum* Avr9/Cf-9 rapidly elicited protein 276 (*ACRE276*) gene (GenBank accession no. AY220483).

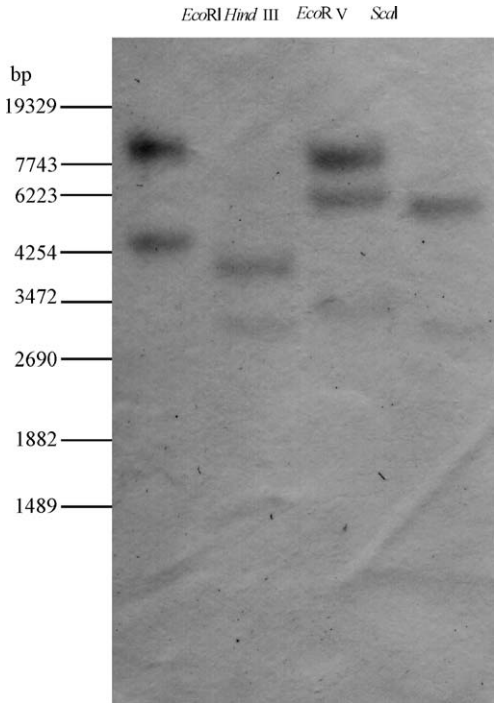

Fig. 2. Southern blot analysis of *StPUB17* genomic copy in potato cv. 386209.10 plants. Fifty micrograms genomic DNA were digested with *Eco*RI, *Hind*III, *Eco*RV and *Sca*I (from left to right) and hybridized under high stringency with DIG labeled full-length *StPUB17* cDNA probe.

Gel blot analyses of potato cv. 386209.10 genomic DNA, probed under high stringency conditions with DIG labeled full-length cDNA of *StPUB17*, which containing one *Eco*R V but no other three restriction enzyme sites, indicated that there were two to three copies of *StPUB17* or closely related genes in the potato genome (Fig. 2).

3.2. The StPUB17 gene encodes a UND/PUB/ARM repeat-domain containing protein

By using the computer pI/Mw tool (http://www.expasy.org), the calculated isoelectric point (pI) and molecular mass of the deduced StPUB17 were inferred to be 8.59 and 79.3 kDa, respectively. Database search with the deduced amino acid sequence suggested that StPUB17 was an UND domain followed by an U-box in the center and ARM repeats in the C-terminal half of the protein (Fig. 3A), which are characteristics of UND/PUB/ARM repeat proteins.

Multiple sequence alignment of StPUB17 and its homologs indicated that StPUB17 was evolutionally closest to the tobacco ACRE276 protein (GenBank accession no. AAP03882) with 89% identity over the entire amino acid sequence. Compared with other *Arabidopsis* and rice UND/U-box/ARM repeat proteins for which full-length cDNAs are available in the public database, StPUB17 was related to the AtPUB17 (GenBank accession no. NP_174228) and *Oryza sativa Japonica Group* putative Avr9/Cf-9 rapidly elicited protein (OsPUB4) (GenBank accession no. BAD27662) with overall 66% and 46% sequence identity, respectively. Moreover, it has 52% identity with *Brassica napus* ARM repeat containing protein (BnARC1) (GenBank accession no. AAB97738) (Fig. 3B). The

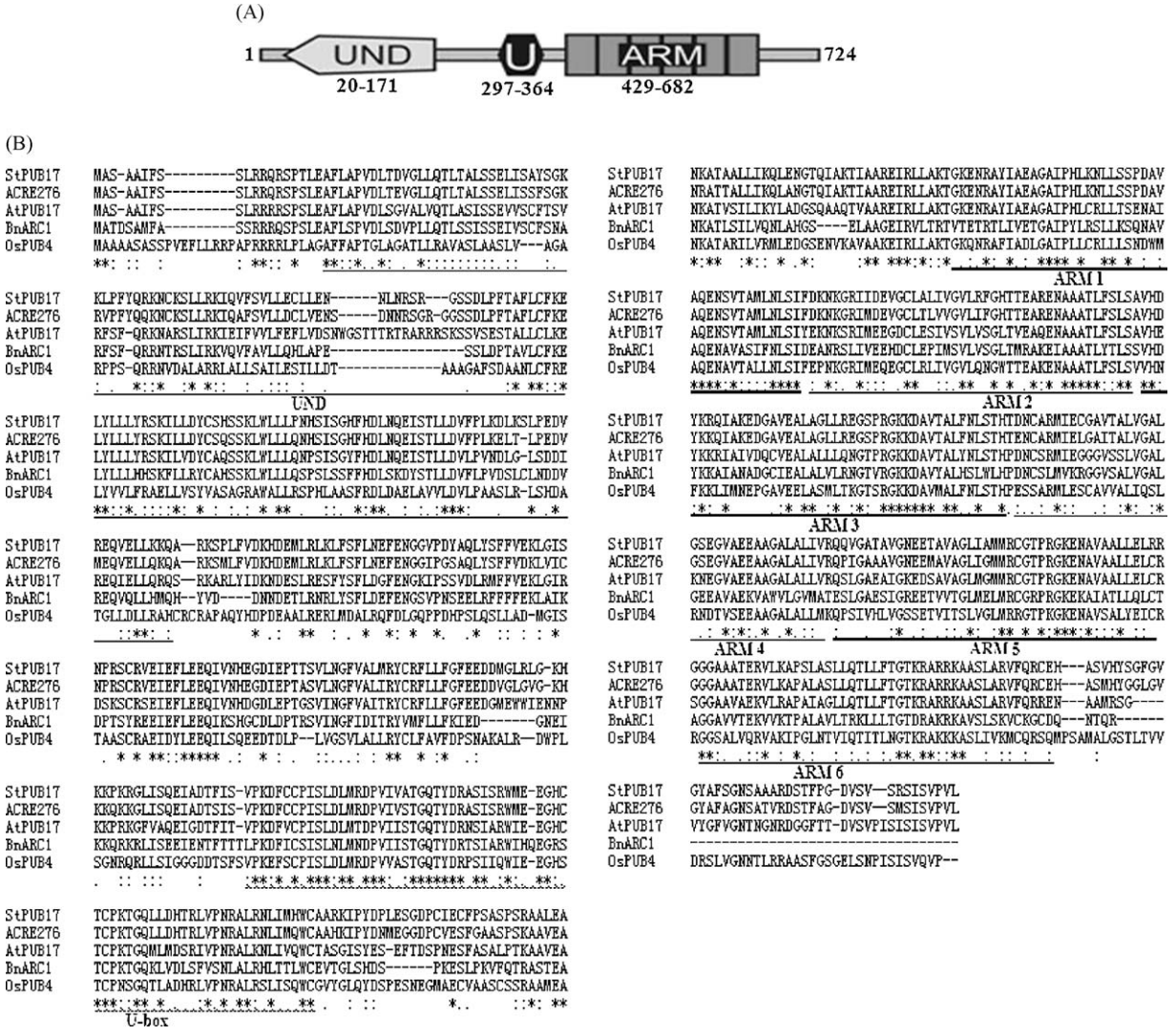

Fig. 3. Protein sequence alignment of potato StPUB17 with related UND/PUB/ARM repeat proteins. Schematic representation of primary structure of putative StPUB17 protein. (A) The UND, U-box and ARM repeat-domains, which are commonly observed in the plant U-box family, are indicated with light gray, black and dark gray boxes, respectively. The numbers show the amino acid position of each conserved domains. (B) Multiple alignment of the amino acid sequences of StPUB17 and other plant U-box proteins. The amino acid sequences deduced from the cDNAs of potato StPUB17, tobacco ACRE276 (GenBank accession no. AAP03882), *Arabidopsis* AtPUB17 (NP_174228), *Brassica* BnARC1 (AAB97738) and rice OsPUB4 (BAD27662) were aligned. Conserved U-box motif, which is essential for E3 ubiquitin ligase activity, UND and ARM repeat-domains, which provide a versatile protein–protein interaction surface used to bind diverse target proteins, are marked by black lines, respectively. (C) Alignment of the derived amino acid sequence of consensus U-box domains among StPUB17 and homologous genes (GenBank accession nos. from top to bottom ABK96801, AAP03882, NP_174228, AAB97738, BAD27662, Q9LZW3, AK099529, BAB93187, BAC98577, BAD26307, Q9XIJ5, O80742, BAB55653, BAD08040, O48700, Q9CAG5, Q9C7G1, BAD82150, CAD41127.2, AY219234, O22193, Q5XZE8, BAD09539, BAD82582, Q8GWV5, Q681N2, BAD61809, BAD10281, Q9C9A6, Q8GUG9, BAD16137, Q8VZ40, BAD67946, Q9ZV31, Q9SNC6, 25815287, AAT94161) using ClustalW software. The dashes "-" denote gaps introduced to maximize the alignment, asterisks "*" show the conserved amino acid residues, and black dots ":" or "." indicate semiconserved amino acids, respectively. (D) Phylogenetic relationship between StPUB17 and other closely related UND/PUB/ARM repeat-domain contained proteins. The phylogenetic tree was generated from the unmodified alignment using ClustalW software.

(C)
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StPUB17  VPKDFCCPISLDLMRDPVIVATGQTYDRASISRWME-EGHCTCPKTQQLLDHTRLVPNRALRNLIMHWC
ACRE276  VPKDFCCPISLDLMRDPVIVSTGQTYDRASISRWME-EGHCTCPKTQQLLDHTRLVPNRALRNLIMQWC
AtPUB17  VPKDFVCPISLDLMTDPVIISTGQTYDRNSIARWIE-EGHCTCPKTQQMLMDSRIVPNRALKNLIVQWC
OsPUB4   VPKEFSCPISLDLMRDPVVASTGQTYDRPSIIQWIE-EGHSTCPNSGQTLADHRLVPNRALRSLISQWC
BnARC1   LPKDFICSISLNLMNDPVIISTGQTYDRTSIARWIHQEGRSTCPKTGQKLVDLSFVSNLALRHLTTLWC
OsPUB2   PPPDFRCPISLDLMRDPVVSASGQTYDRESITRWFG-SGKSTCPKTGQVLANLELVPNKALKNLISRWC
OsPUB3   PPSDFRCPISLDLMRDPVVVASGQTYDRESIDRWFS-SGKSTCPKTGQVLANLELVSNKALKNLISKWC
AtPUB16  IPADFRCPITLELMRDPVVVATGQTYDRESIDLWIQ-SGHNTCPKTGQVLKHTSLVPNRALKNLIVLWC
AtPUB10  IPEDFLCPISLELMKDPAIVSTGQTYERSFIQRWID-CGNLSCPKTQQKLENFTLTPNYVLRSLISQWC
AtPUB11  IPVDFLCPVSLELMKDPVIVATGQTYERAYIQRWID-CGNLTCPKTQQKLENFTLTPNYVLRSLISRWC
OsPUB9   IPEDFRCPISLELMRDPVIVSTGQTYERAFIQRWID-CGNRTCPKTQLKLQNITLTPNYVLRSLILQWC
OsSPL11  IPDEFRCPISLELMKDPVIVSTGQTYERACIEKWIA-SGHHTCPTTQQKMSTSALTPNYVLRSLISQWC
OsPUB10  VPDDFRCPISLDLMKDPVIVATGQTYERGCIERWLE-AGHDTCPKTQQKLPNKSLTPNYVLRSLIAQWC
AtPUB12  PPEEFRCPISLELMTDPVIVSSGQTYERECIKKWLE-GGHLTCPKTQETLTSDIMTPNYVLRSLIAQWC
AtPUB13  IPDDFRCPISLEMMRDPVIVSSGQTYERTCIEKWIE-GGHSTCPKTQQALTSTTLTPNYVLRSLIAQWC
AtPUB14  IPEYFRCPISLELMKDPVIVSTGQTYERSSIQKWLD-AGHKTCPKSQETLLHAGLTPNYVLKSLIALWC
OsPUB12  IPDEFRCPISLELMQDPVIVSSGQTYERSCIQKWLD-SGHKTCPKTQQPLSHTSLTPNFVLKSLISQWC
NtPUB4   IPPDFCCPLSLELMTDPVIVASGQTYERAFIRRWID-LGLTVCPKTRQTLGHTNLIPNYTVKALIANWC
AtPUB4   ILADFFCPLSLEVMTDPVIVSSGQTYEKAFIKRWID-LGLKVCPKTRQTLTHTTLIPNYTVKALIANWC
OsPUB15  IPADFCCPLSLELMSDPVIVASGQTYERVYIKLWLD-EGFTICPKTRQRLGHSNLIPNYTVKALIANWC
OsPUB16  IPADFCCPLSLELMSDPVIVASGQTYEHVFIRKWFD-LGYNICPKTRQILGHTKLIPNFTVKQLIENWC
AtPUB2   VPSDFRCSLSLELMTDPVIVASGQTFERVFIQKWID-MGLMVCPKTRQALSHTTLTPNFIVRAFLASWC
AtPUB3   IPPYFRCPLSTELMLDPVIVASGQTFDRTSIKKWLD-NGLAVCPRTRQVLTHQELIPNYTVKAMIASWL
BgBG55   PPEEYKCPLSKRLMYDPVVIASGQTFERIWIQKWFD-EGNDTCPKTLVKLDHQSLMPNTALKDLISKWC
OsPUB29  PPAEFCCPISTKLMRDPVIITSGQTYERENIERWFR-EGYDTCPRTHIKLENFAMIPNTCMRDLIFNWC
AtPUB6   PPEELRCPISLQLMYDPVIIASGQTYERVCIEKWFS-DGHNSCPKTQQQLPHLSLTPNYCVKGLIASWC
AtPUB7   PPEELRCPISLQLMCDPVIIASGQTYERVCIEKWFS-DGHNTCPKTQQQLPHISLTPNNCVKGLIASWC
OsPUB28  PPEELRCPISLQLMYDPVIISSGQTYERVCIEKWFN-DGHSTCPKTQQQLSHLSLTPNYCVKALIASWC
AtPUB45  PPEELRCPISLQLMYDPVIIASGQTYERICIEKWFS-DGHNTCPKTHQQLSHLCLTPNYCVKALISSWC
OsPUB27  PPEELRCPISLQLMHDPVIIASGQTYERACIEKWFS-SGNTTCPKTRNELSQLSMTPNYCIKGLIASWC
OsPUB13  IPNDFLCPISLEIMTDP-------TYERRSIQKWLD-AGQRTCPKTQQPLGHLSLAPNYALKNLIMQWC
OsPUB14  IPNDFLCPITLAIMRDPVIVATGQTYERRSIQKWLD-SGERTCPKTRQRLSHMSLAPNYALKNLILEWC
AtPUB15  LPHEFLCPITLEIMLDPVIIATGQTYEKESIQKWFD-AGHKTCPKTRQELDHLSLAPNFALKNLIMQWC
OsPUB5   NPEGLQCPITLELMTDPVTLATGQTYDRASIKRWVK-SGCRTCPVTGEKLRSADVVPNVAVRGIVEQLL
OsPUB6   RPEALQCPITLDLMTDPVTVSTGQTYDRASITRWMK-AGCRTCPVTGERLSTADLVPNTVLRGIIERML
AtPUB18  KVEDLLCPISLEIMTDPVVIETGHTYDRSSITKWFG-SGNITCPITGKILTSTELVDNVSVRQVIRKHC
AtPUB19  NVDDLRCPISLEIMSDPVVLESGHTYDRSSITKWFA-SGNITCPKTGKTLVSTVLVDNFSVKQVIQSYS
               *.::  :** **      *::  *  *.   *   ** :   :    .  *  :: .
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(D)

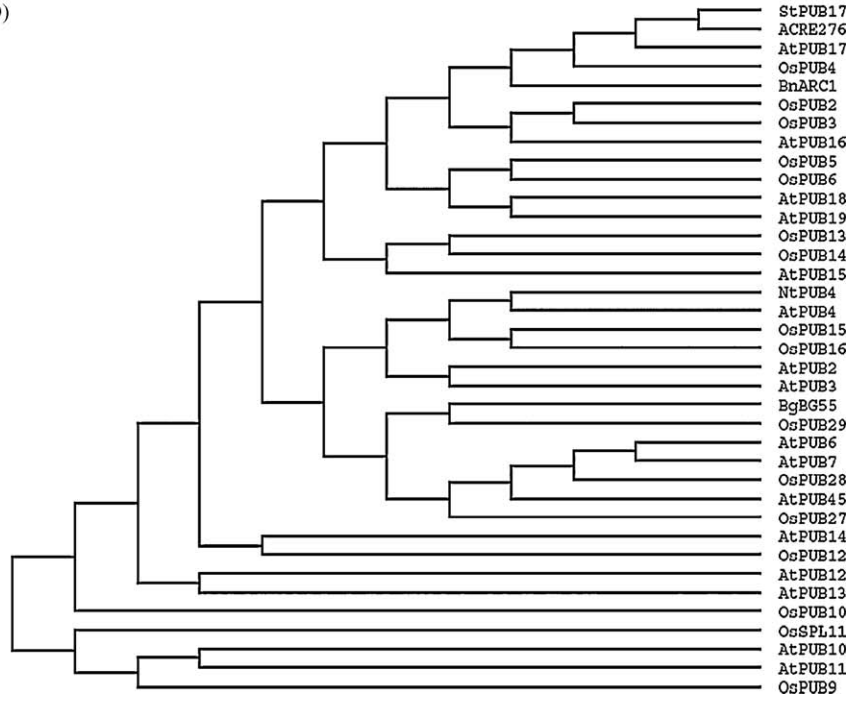

Fig. 3. (*Continued*).

sequence similarity between them was mostly restricted to the regions of UND, U-box and ARM repeats. No significant StPUB17 homolog was identified in human, animals and yeast proteins, suggesting it might be unique to plants.

The sequence analysis for conserved domains revealed that 68 amino acids from position 297 to 364 of StPUB17 shared high similarity to the consensus U-box domain with identity ranging from 67 to 94% (Fig. 3C). In addition, the C-terminal region of StPUB17 showed similarity to the ARM repeats of identified UND/U-box/ARM repeat proteins. In total, six ARM repeat motifs were detected in StPUB17 (Fig. 3A and B).

The phylogenetic relationship among StPUB17 and other members of UND/PUB/ARM repeat family from diverse plant genomes demonstrated that StPUB17 was grouped in the same clade with tobacco ACRE276, Arabidopsis AtPUB17, rice OsPUB4 and *Brassica* BnARC1 (Fig. 3D). Similar results were obtained when the analysis were conducted with other programs (data not shown).

3.3. Expression of StPUB17 gene in different tissues of potato and exposed to the biotic and abiotic stresses

As a first approach to understand the biological function of *StPUB17*, the expression analysis was carried out by RT-PCR with total RNA extracted from various tissues of healthy potato plants. *StPUB17* was constitutively expressed in all tissues examined, whereas the transcripts exhibited much higher levels in leaves and tubers than in roots, stems and flowers (Fig. 4A).

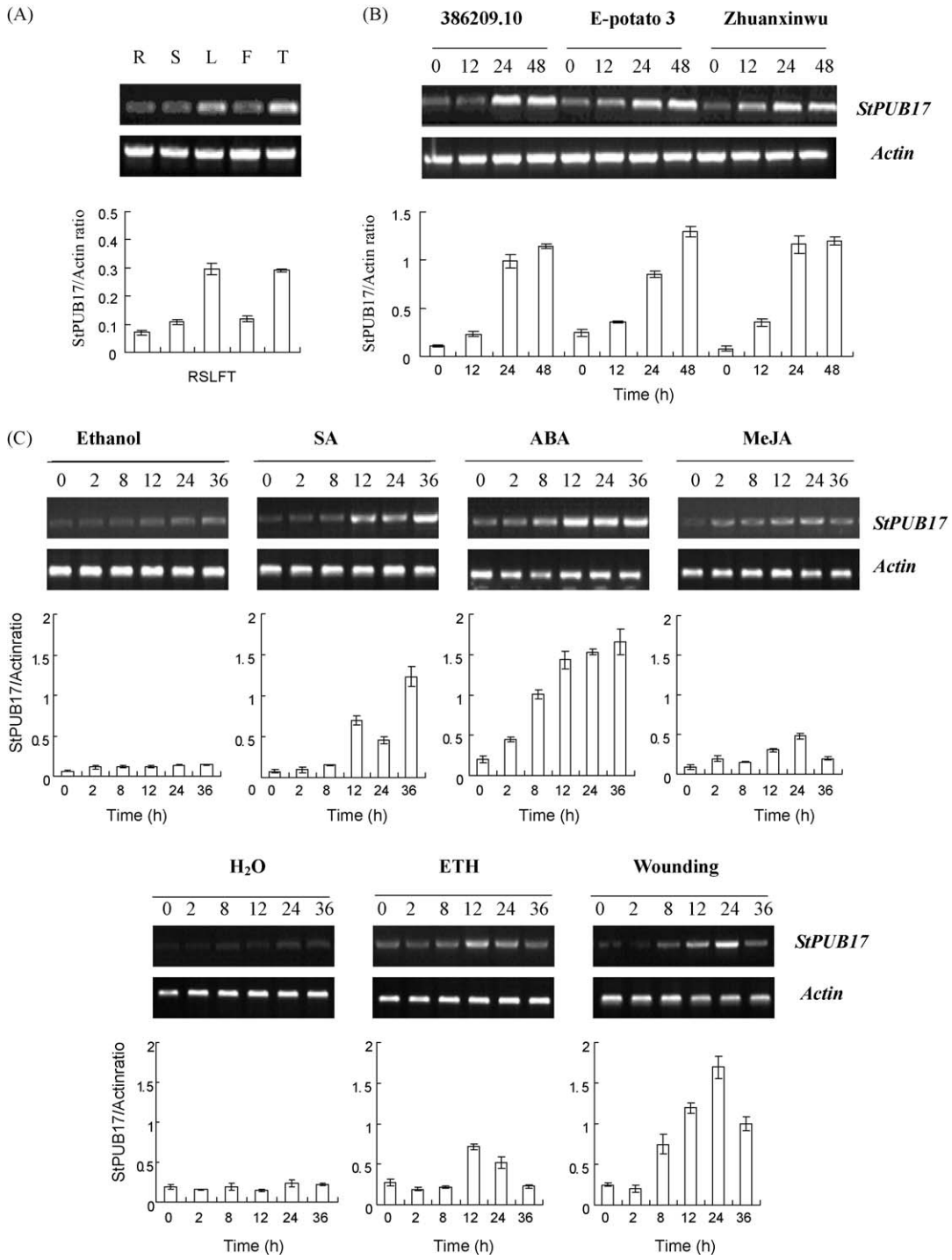

Fig. 4. Levels of potato *StPUB17* gene transcripts in various organs and in response to biotic and abiotic stresses in potato plants. RT-PCR cycles were 28 for A and 27 for B–D. (A) Expression of *StPUB17* gene in different potato organs by RT-PCR analysis. The total RNAs were extracted from roots (R), stems (S), leaves (L), flowers (F) and tubers (T) of potato 386209.10 plants, respectively. (B) RT-PCR analysis of *StPUB17* transcripts in potato detached leaves at various time points after inoculation with the *P. infestans*. Potato cultivars 386209.10 (R-gene-free, horizontal resistance to late blight), E-potato 3 (vertical resistance to late blight) and Zhuanxinwu (susceptible to late blight) were tested. (C) RT-PCR analysis of the *StPUB17* gene expressions in potato 386209.10 detached leaves at various time intervals after treatment with abiotic elicitors: 2 mM SA, 50 μM MeJA, 100 μM ABA or 200 μl l^{-1} ETH and wounding. (D) RT-PCR analysis of the *StPUB17* gene expressions in potato cv. 386209.10 *in vitro* plantlets at various time intervals after treatment with various environmental stresses: 20% PEG, 200 mM NaCl, dehydration, heat (40 °C) and cold (4 °C). The *Actin* gene was used as internal control to show the normalization of the amount of templates in the PCR reactions (lower panel). Relative expression of *StPUB17* mRNA was analyzed by densitometric measurement using Quantity One 1-D Analysis Software (Bio-Rad, USA). The mean density for each band was calculated and expressed as the ratio of *StPUB17* to *Actin*. Error bars indicate standard deviation values of three independent RT-PCR replicates on the same RNA sample.

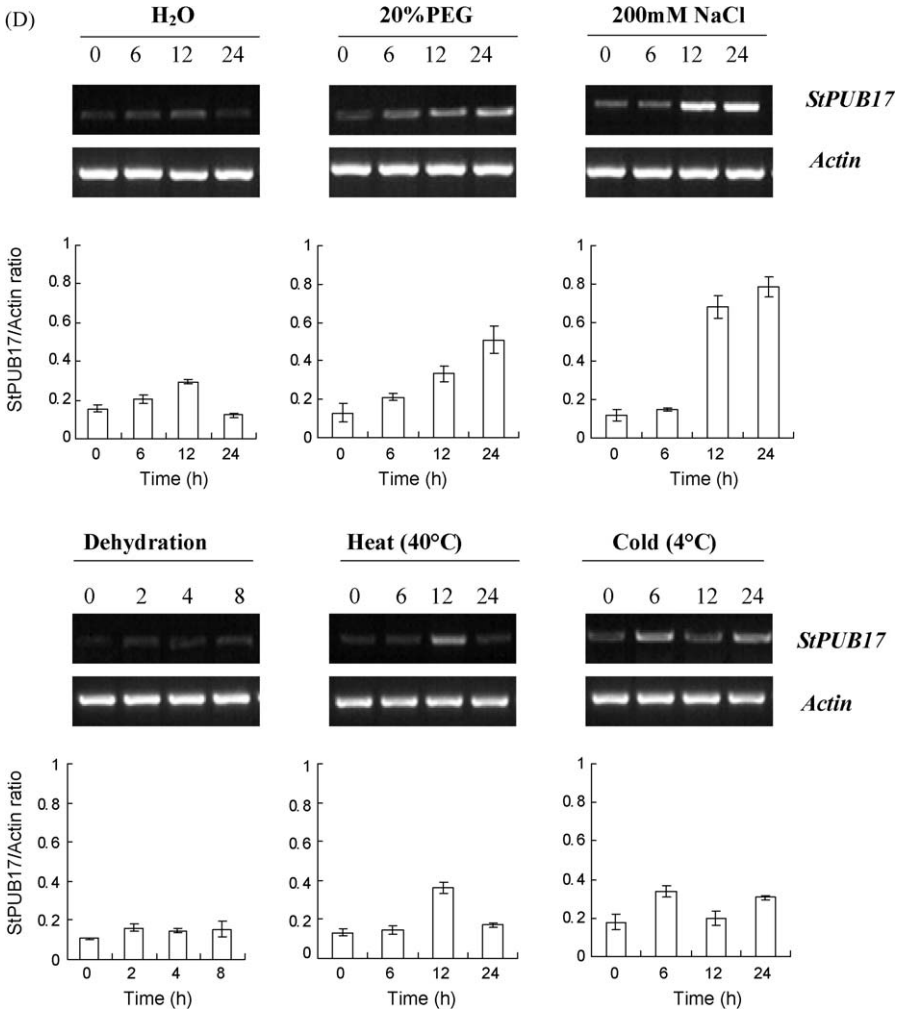

Fig. 4. (*Continued*).

The transcript of *StPUB17* was assayed in detached leaves of three potato cultivars challenged by *P. infestans*. Two days after the pathogen inoculation, the leaves of cv. Zhuanxinwu exhibited water-soaked-like necrosis, E-potato 3 developed hypersensitive responses (HR) and 386209.10 showed no visual symptoms. However, the expression of *StPUB17* was similar in all of the three cultivars, which was induced 12 h after treated, subsequently up-regulated drastically and remained high level till the end of the investigation (48 h) (Fig. 4B).

Plant signal molecules, SA, MeJA, ETH and ABA which have been reported to modulate the expression of biotic and abiotic stress-responsive genes in plants, were applied to detached potato leaves and the induction of *StPUB17* transcripts was monitored (Fig. 4C). SA and ABA induced *StPUB17* expression as early as 8 h after treatment and maintained a relative high level through out the time course, whereas ETH and MeJA induced little detectable accumulation of the transcripts, and even this was late. Mechanical wounding stimulated the gene expression in high abundance with a peak at 24 h and subsequent decline. These results indicated that *StPUB17* may be involved in the stress responses to SA, ABA and wounding.

Furthermore, to elucidate if *StPUB17* is also associated with responses to environmental stresses, *in vitro* plantlets of cv. 386209.10 were subjected to the stresses of cold, heat, osmosis, dehydration and salt, respectively. The results showed that *StPUB17* was responsive to all the stress stimuli but the patterns of expression differed (Fig. 4D). While 20% PEG, dehydration, heat (40°C) and cold (4°C) could induce the accumulation of *StPUB17* mRNA in relatively low abundance, obvious expression signal could be detected after 12 h of treatment with 200 mM NaCl and the expression continued to increase with time. These data also suggested that *StPUB17* may be involved in the pathways responding to environmental stresses and, more importantly, to salt tolerance.

3.4. Generation and molecular characterization of the StPUB17-silenced transgenic plants

In order to provide a more detailed view of the function of *StPUB17* in potato, an *Agrobacterium* transformation vector of *StPUB17*-RNAi was constructed and introduced into cv. E-potato 3. A number of transgenic lines were regenerated on the antibiotic selection medium and the transformation was confirmed by the PCR analysis using specific primers of *NPT* II and 1–3 insertions in potato genome were detected by the Southern blot (data not shown). The efficiency of RNA interference in transgenic lines was determined by semi-quantitative RT-PCR analysis (Fig. 5, upper) which showed that 11 out of 13 independent transgenic lines had over 60% suppression in transcription abundance of *StPUB17* as compared to the control (Fig. 5, bellow), and these lines were selected for further evaluation of the gene function.

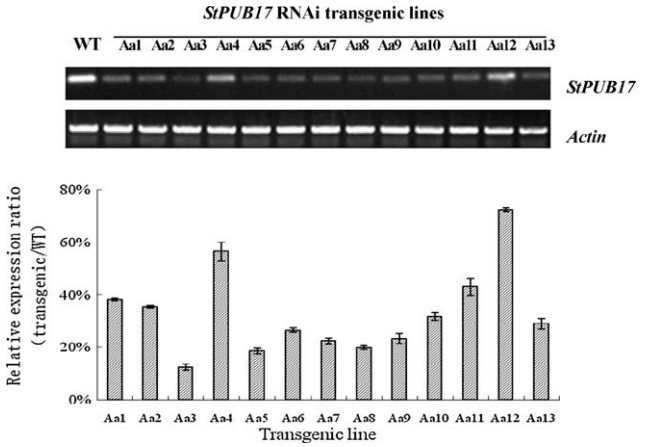

Fig. 5. Analysis of transgenic potato plants silenced *StPUB17* gene. (Upper) RT-PCR analysis showing the expression pattern of *StPUB17* in wild type (WT) plants and transgenic lines using gene-specific primers. The PCR cycles are 30. The *Actin* gene was represented as internal control (lower panel). (Bellow) Quantifying the RT-PCR band intensities in the gel image by percentage of the transcripts of *StPUB17* in individual RNAi transgenic lines to that of the WT control. Aa1–Aa13: *StPUB17* silenced lines of cv. E-potato 3. WT: wild type plants. Error bars indicate standard deviation values of three independent RT-PCR replicates from a pooled sample.

3.5. StPUB17-RNAi potato plants displayed enhanced susceptibility to infection of P. infestans

To test whether the expression of *StPUB17* is associated with potato resistance against the infection of *P. infestans*, the detached leaves of potato *StPUB17*-RNAi lines were challenged with the pathogen zoospores and the lesion growth rate (LGR) was estimated. As illustrated in Fig. 6A, take the transgenic line Aa3 for example, the water-soaked-like necrosis developed on the transgenic leaves 2 days after *P. infestans* inoculation whereas no severe disease symptoms were observed on wild type (WT) plants. The LGR was calculated from lesion radius recorded 3–5 days post-inoculation. The results indicated that the LGR of *StPUB17*-RNAi transgenic plants were higher than the control with Aa3 increase by 45.3% (Fig. 6B). Although there was no significance detected statistically which may resulted from a large variation among replicates, it was virtual that *StPUB17*-RNAi lines had higher susceptibility to *P. infestans* than did the non-transformed control. To further confirm if the expression of the *StPUB17* gene is in accordance with the disease development, the mRNA accumulation profiles under the infection of *P. infestans* were monitored by RT-PCR for the transgenic line Aa3 and the control WT. As shown in Fig. 6C, a much lower and later expression of *StPUB17* was observed in Aa3 than in WT, revealing that silencing of *StPUB17* in potato resulted in a partial loss of plant resistance against late blight.

3.6. StPUB17 is also involved in response to NaCl stress

Since *StPUB17* gene could be rapidly and markedly induced by NaCl (Fig. 4D), its function in the salt tolerance was further investigated by subjecting the *in vitro* plantlets of *StPUB17* RNAi line (Aa3) to different concentrations of NaCl stress. On the MS medium without NaCl, both Aa3 and WT (control) plantlets showed similar development. However, plant growth was obviously retarded by adding NaCl to the medium and the effect becoming stronger as the concentration increased (Fig. 7A). More interestingly, Aa3 plantlets showed much higher sensitivity to NaCl in both 100 mM and 200 mM NaCl than WT in terms of shoot length, plant fresh weight, rooting ratio and percentage of decayed plantlets (Fig. 7B).

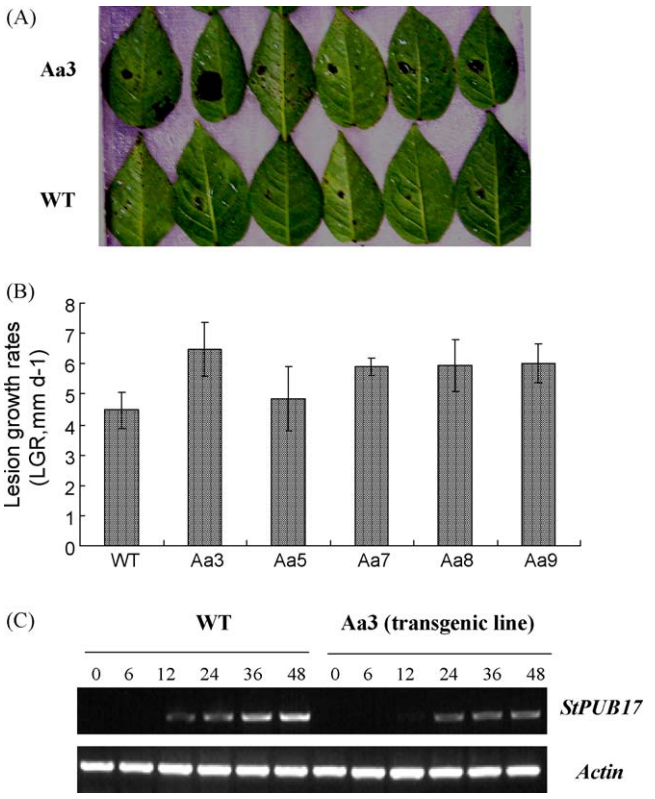

Fig. 6. Response of selected *StPUB17* transgenic lines and WT to *P. infestans* in detached leaves. (A) Symptom developed on the leaves of *StPUB17* transgenic lines (upper) and control (lower) 2 days after inoculation with *P. infestans*. (B) Lesion growth rates (LGR, mm d^{-1}) of *P. infestans* on detached leaves of selected *StPUB17* transgenic lines and relative control plants. Bars indicate the standard error for means of three replications. The significance were tested between the transgenic line and control by *t*-test at $P < 0.05$. (C) Expression analysis of *StPUB17* in transgenic line (Aa3) and control WT in response to the pathogen infection. Potato leaves were collected at various time points (h) after inoculation with the *P. infestans* as indicated on the top of the gel. The transcript accumulation was investigated by RT-PCR assay. The PCR cycles are 26. The *Actin* gene was used as internal control (lower panel).

This phenomenon was also in accordance with the transcription analysis by RT-PCR, when the *in vitro* plantlets were treated with 20 ml 200 mM NaCl solution. The results indicated that NaCl induced *StPUB17* expression 12 h after the treatment and the effect became more pronounced with time in wild type (WT) plantlets. However, the transcripts of *StPUB17* were barely detected in the silenced Aa3 plantlets (Fig. 7C). Present data demonstrated that *StPUB17* was involved in the pathway in response to NaCl stress in potato.

4. Discussion

4.1. StPUB17 is a member of a small and phylogenetically ancient UND/PUB/ARM repeat protein subfamily in potato

In present research, we describe a novel gene of potato, *StPUB17*, characterized by its structure to encode a new member of a small subfamily of U-box proteins. U-box proteins exist in all eukaryotes and comprise of a large protein family that have characteristic sequences outside the U-box domain. Among the plant U-box proteins, a specific subgroup with apparent UND and ARM repeat sequence domains known as UND/PUB/ARM repeat proteins, may interact with the target substrate *via* their ARM repeats or the UND and then recruit other components of the ubiquitination machinery through the U-box [19,33]. So far,

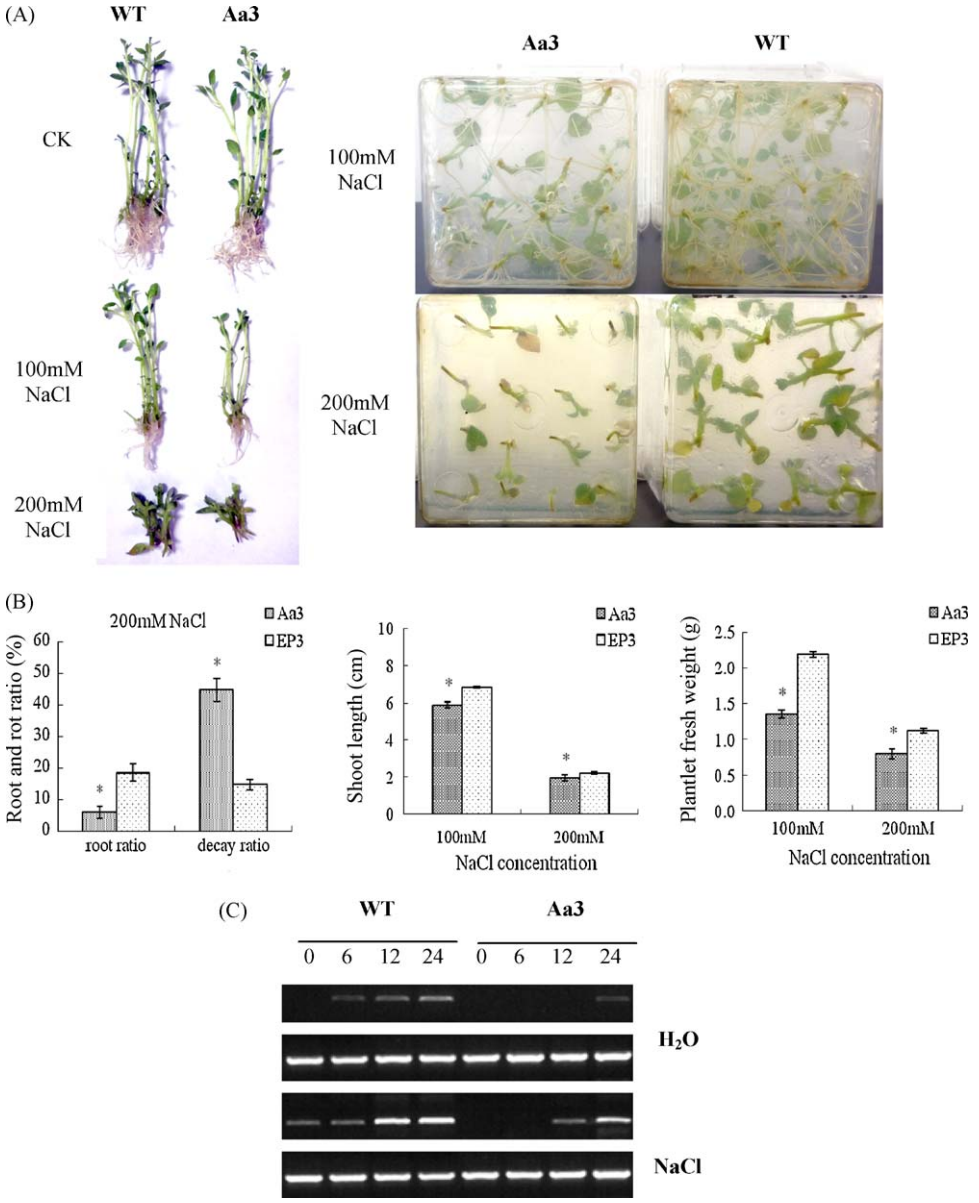

Fig. 7. Salinity susceptivity assays of *StPUB17*-RNAi transgenic (Aa3) and control (WT) plantlets. (A) Phenotype of transgenic and control plantlets after 15 days cultured on MS medium containing 0, 100 and 200 mM NaCl, respectively. (B) Response of *StPUB17* transgenic line in comparison to the control plantlets under NaCl stress. Root (or decay) ratio was calculated by number of plantlets rooted (or stem-end decayed) over the total number of plantlets in each cultured box. Plantlet fresh weight was the total weight of each cultured box. The data are presented as the means ± SE (bar on the top) of six individual measurements, and "*" means significant at $P < 0.05$. (C) Expression analysis of *StPUB17* in transgenic line (Aa3) and control plantlets (WT) under 200 mM NaCl stress. Samples were collected at various time points (h) after treatment as indicated on the top of the gel. The transcript accumulation was investigated by RT-PCR assay. The PCR cycles are 26. The *Actin* gene was used as internal control (lower panel).

thirty-six UND/PUB/ARM repeat proteins have been identified in plant species. The sequence similarity between StPUB17 and other UND/PUB/ARM repeat proteins is mostly restricted to the regions of UND, U-box and ARM repeats (Fig. 3). Database searches revealed that StPUB17 is closest related to the tobacco ACRE276 protein with 89% identity over the entire amino acid sequence, and has 66% identity with *Arabidopsis thaliana* AtPUB17 protein. The U-box and ARM repeat-domains of StPUB17 showed about 94% and 90% identity with those of tobacco ACRE276, respectively. An alignment of StPUB17 with ACRE276, AtPUB17, BnARC1 from *Brassica napus* and OsPUB4 from Rice revealed high similarity in UND and U-box domains and also in the C-terminal region of the proteins (Fig. 3B). Together, characteristics of StPUB17 in sequence demonstrate that StPUB17 is a member of the UND/PUB/ARM repeat protein subfamily in potato.

4.2. The involvement of StPUB17 in defense responses to P. infestans infection

Based on the differentially expressed cDNA clone, *StPUB17* was originally obtained from a suppression subtractive hybridization library constructed from potato 386209.10 leaves which is lack of *R* genes derived from *S. demissum* and possesses resistance to late blight [7]. This suggests that *StPUB17* might be a possible resistance-related gene to *P. infestans*. To clarify the potential biological role of *StPUB17* in the defense responses, the expression pattern of *StPUB17* in potato detached leaves under some well-known defense-related signal molecules including SA, MeJA and ETH were analyzed by RT-PCR. The results showed that *StPUB17* was significantly up-regulated by SA (Fig. 4C). SA has been implicated as a primary component in the signal transduction pathway which may result in resistance of the plants to a variety of pathogens [34,35]. The *StPUB17* gene may,

therefore, be involved in mediating the SA-dependent pathway in the defense responses. More importantly, *StPUB17* was inducible by *P. infestans* in potato cultivars with different late blight resistance types (Fig. 4B). This is in agreement with the notion that the expression of its ortholog *ACRE276* of tobacco is induced in Avr9- and Cf9-mediated incompatible interactions [8]. The pathogen-induced feature during defense responses provides preliminary evidence supporting a role for *StPUB17* in regulating late blight resistance response in potato. Direct evidence supporting this conclusion came from the functional analysis of *StPUB17* in potato transgenic plants. In the present research, *StPUB17* was silenced in a vertical resistance cultivar E-potato 3 by RNAi strategy. If *StPUB17* is indeed required for the full or partial resistance to late blight of this genotype, then silencing this gene should result in increased susceptibility to infection of *P. infestans*. Under the experimental conditions, disease symptom appeared earlier in the transgenic leaves than the non-transgenic control (Fig. 6A). Furthermore, estimation of the lesion growth rate (LGR) indicated that *StPUB17*-RNAi transgenic plants were more susceptive to late blight than the control (Fig. 6B). Our results provide evidences that *StPUB17* is involved in resistance against diverse races of *P. infestans* and that may operate through a SA-dependent defense pathway.

4.3. The involvement of StPUB17 in potato responses to NaCl stress

It has been demonstrated that some of the PUB/ARM proteins play important roles in regulation of abiotic stress tolerance [17,36]. Analysis of the public *Arabidopsis* microarray databases indicates that *Arabidopsis* PUB/ARM genes are expressed in a number of different tissues and under a range of growth conditions, suggesting that these proteins might be involved in a diverse array of responses and developmental processes [37,38]. The *Arabidopsis* AtPUB22, 23 proteins act as negative regulators of both plant defense response and abiotic stress [39,40]. One notable effect is that NaCl treatment increases the expression of several *Arabidopsis* PUB/ARM genes, such as AtPUB6, 7 and 45. These genes are closely related to the mangrove (*B. gymnorrhiza*) BgBG55 that gives mangroves the ability to survive in high salt environments [19,23]. Consistent with these findings the present research showed that the expression of the *StPUB17* gene could be induced in *in vitro* potato plantlets by various external environmental stimuli, including 20% PEG, dehydration, and especially in response to exogenous NaCl (Fig. 4D). This implies that *StPUB17* has a role in mediating ubiquitin-directed protein degradation in adaptation-related responses to various environmental stresses. In *in vitro* potato plantlets, it is evident that the reduced abundance of *StPUB17* transcripts is related to potato salinity sensitivity (Fig. 7), suggesting that *StPUB17* is also involved, in addition to late blight resistance, in response to the salt stress.

5. Conclusion

The *StPUB17* gene encodes a putative E3 ubiquitin ligase activity-related protein which is a novel member of the UND/PUB/ARM repeat protein subfamily in potato. This gene has a broad of spectrum responses to both biotic and abiotic stresses in potato, and is clearly involved in late blight resistance and NaCl susceptibility. Further work should deepen our understand of the biological roles of this potato UND/PUB/ARM protein by the biochemical assays of the E3 ubiquitin ligase activity of *StPUB17*, identification of its upstream and downstream partners, and elucidation of the mechanisms under which it works in the cell.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2009.12.002.

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